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60 kDa Lysophospholipase, a New Sgk1 Molecular Partner Involved in the Regulation of ENaC

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Key Words
Sgk1 • 60 KDa Lysophospholipase • Yeast two hybrid • Erk • EnaC • Sodium Transport

Abstract
The serum- and glucocorticoid-regulated kinase (Sgk1) is essential for hormonal regulation of ENaC-mediated sodium transport and is involved in the transduction of growth-factor-dependent cell survival and proliferation. The identification of molecular partners for Sgk1 is crucial for the understanding of its mechanisms of action. We performed a yeast two-hybrid screening based on a human kidney cDNA library to identify molecular partners of Sgk1. As a result the screening revealed a specific interaction between Sgk1 and a 60 kDa Lysophospholipase (LysoLP). LysoLP is a poorly characterized enzyme that, based on sequence analysis, might possess lysophospholipase and asparaginase activities. We demonstrate that LysoLP has indeed a lysophospholipase activity and affects metabolic functions related to cell proliferation and regulation of membrane channels. Moreover we demonstrate in the *Xenopus* oocyte expression system that LysoLP downregulates basal and Sgk1-dependent ENaC activity. In conclusion LysoLP may represent a new player in the regulation of ENaC and Sgk1-dependent signaling.

Introduction

The serum- and glucocorticoid-induced kinase Sgk1 is a serine/threonine kinase that regulates sodium absorption by the amiloride-sensitive epithelial sodium channel (ENaC) in principal cells of the distal nephron [1, 2]. The kinase is upregulated at the transcriptional level and/or activated by a wide variety of hormones [3, 4]. It is also considered an important molecular target that integrates multiple endocrine inputs in the regulation of several ion channels (ENaC, TRPV5, ROMK, KCNE1/KCNQ1, ClCKa/Barttin), carriers (NCC, NKCC, NHE3, SGLT1, EAAT1-5) and the Na^+/K^+-ATPase [5-7].

The steroid hormone aldosterone increases the synthesis of Sgk1, whereas activation of the enzyme is dependent on phosphorylation. PDK2 (phosphoinositide-dependent kinase 2) has finally been identified as mTOR and found to be the H-motif kinase that phosphorylates

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Sgk1 at S422 to prime it for phosphorylation by PDK1 at T256 [8]. Sgk1 is also involved in mediating growth factor-, insulin-, IL-2- and steroid-dependent survival signals [9, 10].

ENaC activity is also down regulated by the activation of the mitogen-activated protein kinases ERK 1/2 in response to stimuli as different as lipopolysaccharides [11] or PKC [12].

Our laboratory has recently demonstrated that Sgk1 phosphorylates and activates MDM2, driving p53 to proteosomal degradation, thus disclosing a possible mechanism by which Sgk1 protects cells from stress-dependent apoptosis [13].

We have also used yeast two-hybrid screening to identify Sgk1-binding partners. We characterized the interaction of Sgk1 and phosphomannomutase 2 (PMM2), showing that PMM2 activity is inhibited by Sgk1 [14]. The identification of other Sgk1-interacting molecular partners may be important for understanding the mechanisms inducing channel regulation and transduction of survival signals.

In the present paper we describe a novel Sgk1-interacting protein known as *60 kDa Lysophospholipase* (from now on referred to as LysoLP) that, based on sequence data, is expected to possess lysophospholipase and asparaginase activities [15]. We present evidence of physical interaction between LysoLP and Sgk1. Moreover, we demonstrate that LysoLP increases ERK1/2 phosphorylation and has a measurable effect on the metabolism of lysophospholipids. Finally, we show, in *Xenopus* oocytes, that the expression of LysoLP decreases membrane expression of ENaC and inhibits basal and Sgk1-dependent activation of ENaC measured by two-electrode voltage-clamping.

Taken together, our data suggest that LysoLP can be considered a new player in Sgk1-dependent regulation of channel and transporter activities.

Materials and Methods

Constructs

pBridge-2.1 and *pBridge-4.1*. Sgk1 mutants 2.1 (inactive) and 4.1 (dominant negative) [4, 5] were cloned into pBridge by means of a PCR-based method, using primers with EcoRI and BamHI restriction sites according to previously published methods [14]. pcDNA4/TO/Myc-Sgk1 was used for transfection in mammalian cells [9].

pCMV-HA-LysoLP. LysoLP, from pACT2-LysoLP was subcloned into pCMV-HA using the EcoRI-XhoI restriction sites of the pACT2-LysoLP, selected from the human kidney library, and used for the yeast two-hybrid assay (BD Biosciences/Clontech, Palo Alto, CA).

pACT2-LysoLP(Ank). A deletion mutant of LysoLP, containing the ankyrin repeats, lacking of the first 1265 bp at the N-terminal portion of LysoLP, was cloned in pACT2. The coding sequence was amplified by Klen *Taq* polymerase, using pACT2-LysoLP as a template. Primers introducing EcoRI (CCG AAT TCG AGA GGC AGA TGC CCT GCG GAA T) and XhoI (GTG AAC TTG CGG GG TTT TTC AG) restriction sites were used.

pGEX-4T-3-LysoLP. The full-length human LysoLP cDNA was cloned in pGEX-4T-3 (Amersham Biosciences, Freiburg, Germany). The coding sequence of LysoLP, was amplified by PCR, using pACT2-LysoLP as template. Primers introducing EcoRI (CCG AAT TCC ATG GCG CGC GCG GTG GGG) and XhoI (GAC TCG AGC TTA GAC ACC AGG CAG CAC) restriction sites were used.

pcDNA3-LysoLP. The amplified coding sequence of LysoLP (see above), was also subcloned into pCDNA3 at EcoRI-XhoI restriction sites.

pcDNA3 HA-B-Raf and pcDNA3 HA-B-Raf S364A were kindly provided by Kun-Liang Guan University of Michigan, Ann Arbor, Michigan, USA.

pEF Myc-B-Raf was kindly provided by Massimo Santoro, Federico II University, Napoli, Italy.

Yeast Two-Hybrid Screening

A yeast two-hybrid system assay was performed (MATCHMAKER Gal4 Two-Hybrid; BD Biosciences/Clontech, Palo Alto, CA). The plasmid pBridge-Sgk1 expressing a fusion protein with GAL4 DNA binding domain and human full-length Sgk1 was used as bait. The detailed protocol was described in a previous study [14]. The cDNA encoding specific Sgk1-interacting proteins were sequenced and studied with BLAST analysis. Specificity of the interaction was tested by AH109 co-transformation of the selected plasmid with pBridge-Sgk1 and two unrelated baits (pBridge-Adducin and pBridge empty vector).

Interaction assay in yeast

Transformed yeast cells were grown in selective medium for 24 h and then harvested. Cells were lysed, and ONPG substrate was added. OD was determined by a spectrophotometer. This ONPG/β-Gal assay is referred to as the "Miller" assay, and a standardized amount of β-Gal activity (a "Miller Unit"), is calculated as:

$$1000 * \frac{(Abs_{420})}{((Abs_{600} \text{ of culture sampled}) * (\text{volume [0.02 mL]}) * (\text{reaction time}))}$$

Cells and transfections

COS7 monkey kidney cells and 293T modified human embryonic kidney fibroblasts (HEK-293T) cells were grown in DMEM containing 10% fetal bovine serum at 37°C with 5% CO_2. Cells were plated at 70-80% density in six-well plates. On the next day they were transfected with expression plasmids (600 ng/well) using Lipofectamine plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Twenty four hours following transfection, the medium was

replaced with serum-free medium (DMEM containing 0.1% insulin-free bovine serum albumin plus antibiotics).

Co-immunoprecipitation Experiments

Co-transfected cells were lysed in a solubilization buffer containing 50 mM Tris HCl pH 7.8, 300 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 10 mM NaF, 5 mM sodium pyrophosphate, 2 mM sodium orthovanadate and complete protease inhibitors (Complete Roche Molecular Biochemicals, Mannheim, Germany). Protein extracts were quantified by a Bradford-based assay and immunoprecipitated with the co-immunoprecipitation kit (Pierce, Rockford, IL) following the manufacturer's instructions. Immunoprecipitates were then subjected to Western blot analysis.

Western blot analysis

Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Filters were blocked in 5% non-fat dry milk, incubated with the appropriate primary antibodies, washed three times, and incubated with the secondary antibodies. Protein bands were detected by ECL. Primary antibodies used were: anti-HA and anti-Myc (diluted 1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA,USA), and anti-p-ERK1/2 (diluted 1:1000) and anti-ERK (diluted 1:10000) from Cell Signaling Technology (Beverly, MD).

Preparation of GST-LysoLP

The bacterial strain *BL21* was transformed with the pGEX-4T-3-LysoLP construct. Bacteria were exponentially grown at 37°C for 2 h and subsequently induced with 0.5 mM isopropyl-1-thio-D-galactopyranoside for 3 h at 30°C. Cells were washed, resuspended in a lysis buffer containing 1% Triton X-100, 0.1% glycerol, and 1 mM dithiothreitol, in phosphate-buffered saline (PBS) supplemented with protease inhibitors (Complete Roche Molecular Biochemicals, Mannheim, Germany). The GST-LysoLP fusion protein was subsequently purified by glutathione Sepharose beads (Pharmacia/Pfizer) and dialyzed overnight at 4°C against a buffer containing 50 mM Tris HCl pH 7.5, 1 mM EDTA and 50 mM NaCl. The concentration of GST-LysoLP was calculated by the Bradford method and verified by SDS-PAGE and Coomassie blue staining.

Pull-down assay

GST-LysoLP bound to Sepharose beads was mixed with 500 µg of protein extracts of COS7 cells transiently transfected with pcDNA4/TO/Myc-Sgk1 in a buffer containing 20 mM Tris HCl 7.4, 0.1 mM EDTA, 100 mM NaCl, 0.5% NP40. The reaction mixture was incubated for 1 h at room temperature. After extensive washing with buffer, the beads were incubated for 10 min in Laemmli buffer, and the supernatant was electrophoresed on a 12% polyacrylamide gel and analyzed by Western blotting.

Immunofluorescence

Forty eight hours following transfection, cells were washed twice with PBS and fixed for 15 min in 4% paraformaldehyde in PBS. Fixed cells were washed twice with PBS and then permeabilized for 5 min with 0.2% Triton X-100 in PBS. Permeabilized cells were incubated in a humidified chamber at room temperature with primary antibodies (rabbit polyclonal anti-Myc, Santa Cruz Biotechnology, Santa Cruz, CA and mouse monoclonal anti-HA, Roche Diagnostics, Indianapolis, IN) diluted 1:200 in PBS for 1 h, washed twice with PBS, incubated for 45 min with an appropriate secondary antibody (Alexa Fluor 488 goat anti-rabbit IgGs and Alexa Fluor 568 donkey anti-mouse IgGs from Molecular Probes, Eugene, OR), diluted 1:800 in PBS, washed twice with PBS, and mounted with Prolong antifade reagent (Molecular Probes). Visualization was achieved using a confocal microscope (Leica Microsystems, Wetzlar, Germany).

Lysophospholipase activity

HEK293T cells transfected with pCMV-HA or HA-LysoLP were distrupted by sonication in a lysis buffer containing 20 mM Tris HCl pH 7.5, 0.1 mM EDTA, 2 mM β-mercaptoethanol, 1 mM DTT, 10% glycerol and complete protease inhibitors (Sigma, St. Louis, MO) as reported before [13]. Cell lysates were centrifuged at 200 x g for 5 min at 4°C and the postnuclear supernatants further centrifuged at 10,000 x g for 20 min at 4°C. The final membrane pellet was resuspended in lysis buffer. Ninety µg of membranes were incubated with 0.2 mM LysoGroPIns from bovine liver (Avanti Polar Lipids, Alabaster, AL) and 15,000 cpm/sample of [^{3}H]-LysoGroPIns in 20 mM sodium phosphate pH 6, for 2 h at 37°C.

The incubations were terminated by addition of cold methanol (-20°C), followed by two-phase extraction, lyophilization of the resultant upper (aqueous) phase and HPLC analysis [16].

Kinase assay

HEK293T cells transfected with or without LysoLP were stimulated with insulin (1 µmol) for 30 min. Cell monolayers were then solubilized, and an aliquot (500 µg) was immunoprecipitated with rabbit anti-Sgk1 antibody (7 µl). An artificial substrate peptide (Arg-Pro-Arg-Thr-Ser-Thr-Phe, 1 mM) was used for the immune complex kinase assay (1 mM), as previously reported [4]. Background incorporation of radioactivity was estimated by assaying extracts prepared from untransfected cells incubated in the presence or absence of insulin. To determine the kinase activity of Sgk1, the average level of background ^{32}P incorporation was subtracted from ^{32}P incorporation catalyzed by extracts of transfected cells.

Oocyte preparation; injection of cRNA and electrophysiological recordings

Electrophysiological recordings were performed using two-electrode voltage clamp techniques. cRNAs encoding the rat ENaC α, β and γ subunits (generously provided by B. Rossier, Lausanne, Switzerland), human Sgk1 [17], and LysoLP cRNA were synthesized *in vitro* [18]. Oocytes were injected with 15 ng human Sgk1, 10 ng LysoLP cRNA or H_2O on the first day after preparation of the oocytes. One day later, 1 ng/subunit rat wild-type ENaC α, β and γ subunits cRNA were injected. All experiments were performed at room temperature 4 days after injections. Two-electrode voltage-clamp recordings were performed at a holding potential of -80 mV [19]. The data were filtered at 10 Hz and recorded with a GeneClamp 500 amplifier, a

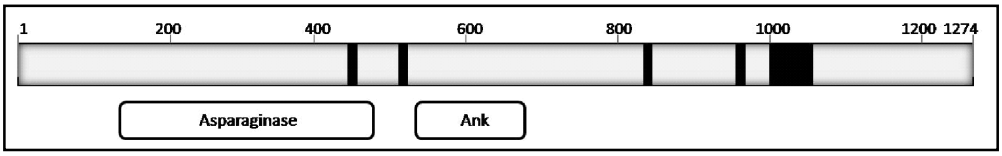

Fig 1. Predictive structure of 60 kDa lysophospholipase. The amino-terminal asparaginase domain and the carboxy-terminal ankyrin domains are boxed.

DigiData 1322A A/D-D/A converter, and the pClamp 9.0 software package for data acquisition and analysis (Axon Instruments, USA) [20]. The bath solution (superfusate/ND96) contained 5 mM HEPES pH 7.4, 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$ and 1 mM $MgCl_2$. The amiloride-sensitive current was measured with 50 μM amiloride for 2 min. Epithelial sodium channel currents were calculated as the difference in whole-cell current before and after the addition of amiloride to the bathing solution. The flow rate of the superfusion was 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s. Data are provided as means ± SEM, for n oocytes investigated. All experiments were repeated with at least three batches of oocytes; in all repetitions qualitatively similar data were obtained.

Western Blot of Plasma Membrane Proteins

The membrane abundance of Flag-βENaC was analyzed by surface biotinylation. Twenty intact healthy oocytes were incubated in Sulfo-NHS-LC-Biotin (1 mg/ml, Pierce, Rockford, IL, USA) for 30 minutes at room temperature and washed 5 times for 10 min in ND96. The oocytes were homogenized with a pestle in 400 μl buffer H (20 mM Tris HCl, pH 7.4, 100 mM NaCl, 1% TritonX-100 and Complete Protease Inhibitor [(Roche Diagnostics GmbH, Mannheim, Germany]). The samples were kept at 4°C for 1 h on a rotator, and then centrifuged for 1 min at 13,000 rpm. The supernatants were supplemented with 25 μl washed immobilized NeutrAvidin Agarose beads (Pierce, Rockford, IL, USA) and incubated at 4°C overnight on a rotator. The beads were then pelleted by a 2 min centrifugation at 13,000 rpm, and washed in buffer H (5 times). Proteins were separated on a 10% polyacrylamide gel and transferred to PVDF membrane. After blocking with 5% non-fat dry milk in TBS 0.1% Tween20 for 1 h at room temperature, the blots were incubated overnight at 4°C with mouse anti-Flag M2 antibody (diluted 1:1,000, Sigma Aldrich, Saint Louis, Missouri, USA). After washing (TBST), the blots were incubated with anti-mouse HRP-conjugated antibody (diluted 1:1,000, Cell Signaling, Danvers, MA, USA) for 1 h at room temperature. Antibody binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany). Bands were quantifed with Quantity One Software (Bio-Rad, München, Germany).

Results

Identification of the 60 kDa lysophospholipase as an Sgk1 molecular partner

The two-hybrid screening resulted in the isolation of a yeast clone containing an ~1700 bp cDNA. Other clones selected by the screening were previously reported [12].

Beta Gal analysis	
Interactors	Miller Units
Sgk1/LysoLP	67 ± 0.7
2.1/LysoLP	68 ± 2.4
4.1/LysoLP	68 ± 1.4
Sgk1/LysoLP(Ank)	6 ± 0.7
Sgk1/PMM2	66 ± 2.6
pBrigde/LysoLP	1 ± 0.4

Table 1. Interaction assay in yeast

BLAST analysis of the 1700-bp-cDNA showed that the isolated contains the full-length sequence coding for a protein named *60 kDa Lysophospholipase* (LysoLP) (NP_001073933). Specificity of the interaction was demonstrated by re-transforming the selected clone with the bait (pBridge-Sgk1) and two other unrelated baits (pBridge-Adducine and pBridge empty vector). Only co-transformation of pACT2-LysoLP with pBridge-Sgk1 allowed the growth of the yeast in selective media.

The gene encoding LysoLP maps at the position 14q32.33. LysoLP is a protein with a predicted asparaginase domain in the amino-terminal region and four ankyrin domains in the carboxy-terminal region (Fig. 1).

The strength of the interaction between Sgk1 and LysoLP was measured by means of a β-galactosidase assay. Sgk1 kinase activity was not necessary for the interaction with LysoLP, since both kinase dead (S422A) (clone 2.1) and dominant negative (D222A) (clone 4.1) Sgk1 mutants were as effective as the wild-type in the interaction with LysoLP (Table 1). An amino terminal deletion mutant of LysoLP, LysoLP(Ank) containing the four ankyrin domains, was generated. When tested for the interaction with Sgk1, turned out to be significantly less efficient than the full-length LysoLP (Table 1).

Sgk1/LysoLP interaction in mammalian cells

Myc-tagged Sgk1 and HA-LysoLP-expressing vectors were co-transfected in COS7 cells. Proteins were extracted and subjected to immunoprecipitation as de-

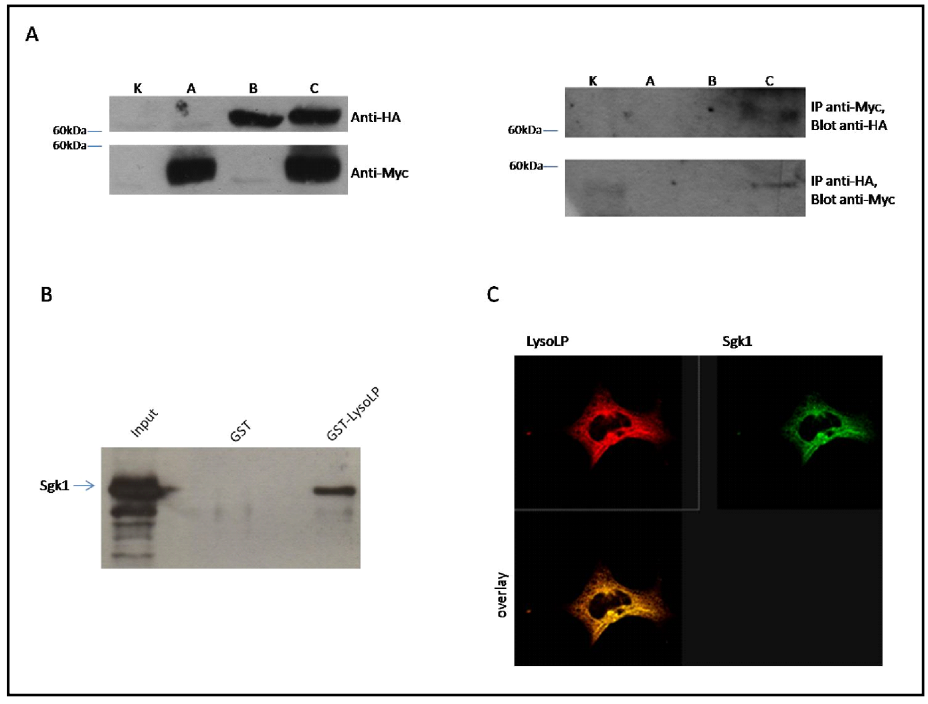

Fig 2. Sgk1 and LysoLP interaction. A: Sgk1 and LysoLP co-immunoprecipitation. COS7 cells were transfected with empty vector (lane K), pcDNA4/TO/Myc-Sgk1 (lane A), pCMV-HA-LysoLP (lane B) and both pcDNA4/TO/Myc-Sgk1 and pCMV-HA-LysoLP (lane C). Protein extracts (left panels) and anti-Myc or anti-HA immunoprecipitates (IP, left panels) were analyzed by SDS-PAGE and revealed by immunoblotting with anti-HA (upper panels) and anti-Myc immunoglobulins (lower panels) as indicated. B: Sgk1 and GST-LysoLP pull-down assay. Cell lysates from COS7 cells transfected with pcDNA4/TO/Myc-Sgk1 were allowed to bind a resin containing recombinant GST or GST-fused LysoLP. Myc-tagged Sgk1 was detected in SDS eluates by immunoblotting with anti-Myc antibodies. C: Co-localization of Sgk1 and LysoLP. Monolayers of COS7 cells grown on glass coverslips were transfected with expression vectors encoding the full-length Myc-tagged Sgk1 (pcDNA4/TO/Myc-Sgk1) and the full-length HA-tagged LysoLP (pCMV-HA-LysoLP). Serum-starved cells were fixed as indicated in materials and methods and stained with rabbit anti-Myc immunoglobulins to visualize Sgk1 or with mouse anti-HA immunoglobulins to visualize LysoLP. The staining for Sgk1 was detected with Alexa Fluor 488 goat anti-rabbit IgGs. The staining for LysoLP was detected with Alexa Fluor 568 donkey anti-mouse IgGs.

scribed in the section of materials and methods. Co-immunoprecipitation of LysoLP with anti-Myc Sepharose beads was observed only in the presence of Sgk1. Co-immunoprecipitation of Sgk1 with anti-HA Sepharose beads was observed only in the presence of LysoLP (Fig. 2 A).

The interaction of LysoLP and Sgk1 was confirmed by a pull-down assay. Pull-down experiments were carried out by applying protein extracts from COS7 cells transiently transfected with pcDNA4/TO/Myc-Sgk1 to columns containing either recombinant purified GST or GST-fused LysoLP. Myc-Sgk1 was detected by Western blotting with anti-Myc immunoglobulins only in the eluates obtained from the GST-LysoLP columns (Fig. 2 B).

Finally, extensive co-localization of Sgk1 and LysoLP in the cytoplasm of co-transfected COS7 cells was observed (Fig. 2. C).

Lysophospholipase assay

An *in vitro* lysophospholipase assay was carried out using [^{3}H]-lysoglycerophosphocholine (LysoGroPCho) and [^{3}H]-lysoglycerophosphoinositol (LysoGroPIns) as

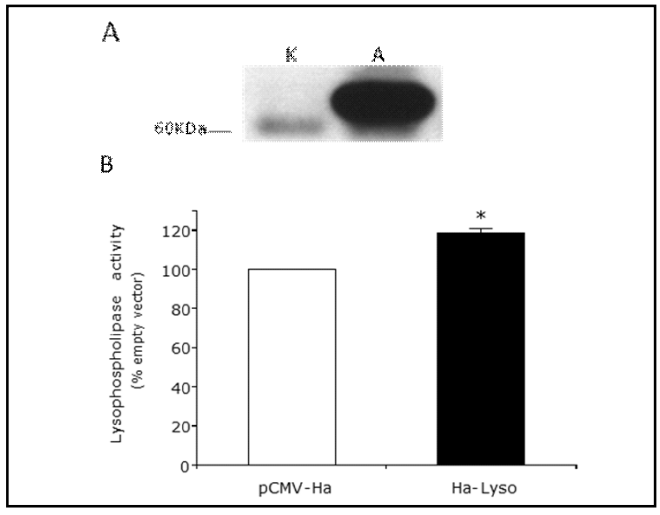

Fig 3. Lysophospholipase activity. A: HEK 293T cells were transfected with either empty vector (lane K) or pCMV-HA-LysoLP (lane A). Proteins extracts were analyzed by SDS-PAGE and revealed by immunoblotting with anti-HA. B: Lysophospholipase activity was measured as radioactivity associated with GroPIns recovered after 2-h-incubation of 90 ug membrane proteins from either pCMV-HA (open bar) or HA-LysoLP-transfected cells (closed bar) with 0.2 mM LysoGroPIns and 15,000 cpm/sample of [^{3}H]-LysoGroPIns in 20 mM sodium phosphate pH 6 (see also materials and methods). Data are expressed as percentage of empty-vector-transfected membranes and are mean ± SEM of 5 experiments in duplicates. * ($p<0.05$).

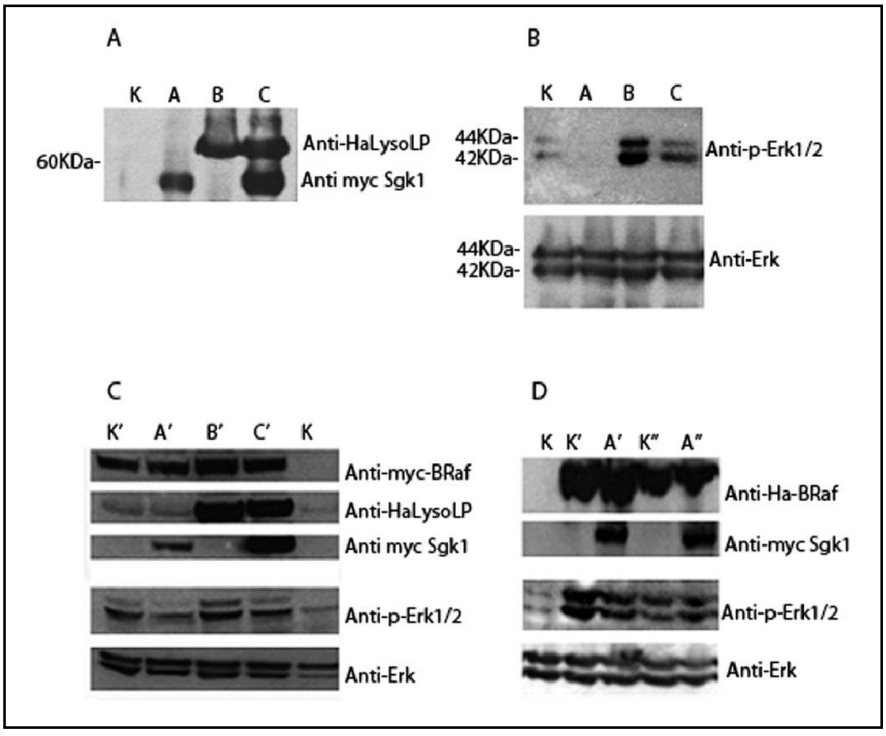

Fig 4. ERK1/2 phosphorylation. A: HEK 293T cells were transfected with empty vectors (lane K) or vectors coding for wild-type Myc-Sgk1 (laneA), HA-LysoLP (laneB), or both wild-type Myc-Sgk1 and HA-LysoLP (lane C). Cell extracts were separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with anti-HA and/or anti-Myc immunoglobulins as indicated. B: Blots prepared from cell extracts as described in panel A were incubated with phospho-ERK immunoglobulins (panel B, top) and ERK immunoglobulins for normalization (panel B, bottom). The results are representative of three independent experiments. C: HEK 293T cells were transfected with empty vectors (lane K) or vectors coding for wild-type Myc B-Raf (lane K'), wild -type Myc B-Raf and Myc-Sgk1 (laneA'), wild -type Myc B-Raf and HA-LysoLP (laneB'), or wild-type Myc B-Raf, wild-type Myc-Sgk1 and Ha-LysoLP (lane C'). Cell extracts were separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with anti-HA and/or anti-Myc immunoglobulins, phospho-ERK immunoglobulins and ERK immunoglobulins as indicated. D: HEK 293T cells were transfected with empty vectors (lane K) or vectors coding for wild-type HA-B-Raf (lane K'), wild -type HA-B-Raf and Myc-Sgk1 (lane A'), S364A-HA-B-Raf mutant (Lane K'') or S364A-HA-B-Raf mutant with wild-type Myc-Sgk1 (lane A''). Cell extracts were separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with anti-HA and/or anti-Myc immunoglobulins, phospho-ERK immunoglobulins and ERK immunoglobulins as indicated.

substrates, in the presence of cell membranes purified from HEK293T cells transfected and not with vectors coding for HA-LysoLP. The overexpression of LysoLP was associated with a modest (~20%) but still significant increase in the lysophospholipase activity detected in cell membranes toward LysoGroPIns (Fig. 3), while no activity was monitored in presence of LysoGroPCho. This lysophospholipase activity was not affected by the co-expression of Sgk1 in HEK293T cells, suggesting that the interaction between LysoLP and Sgk1 has no effect on the lysophospholipase activity associated with LysoLP.

The effects of Sgk1 and LysoLP expression on ERK1/2 phosphorylation

In HEK293T the transient transfection with the vector coding for wild-type Sgk1 was associated with a sharp decline in the phosphorylation of ERK1/2 (Fig. 4 B, lane A) . On the contrary, in HEK293T cells transiently transfected with LysoLP a clear-cut increase in the phosphorylation of ERK1/2 was detected, (Fig. 4 B, lane B). The co-transfection of both LysoLP and Sgk1 resulted in a level of ERK1/2 phosphorylation that ranked in between the stimulatory effect of LysoLP and the inhibitory effect of Sgk1 (Fig. 4 B , lane C).

Sgk1 has previously been shown to negatively regulate the Raf pathway [21]. Transfection of wild type B-Raf [22], strongly enhanced ERK1/2 phosphorylation (Fig. 4 C, lane K'). The effect of Sgk1 and LysoLP on ERK1/2 phosphorylation was also maintained in HEK293T cells over-expressing wild type B-Raf thus suggesting that Sgk1 acts on both endogenous and exogenous B-Raf (Fig. 4 C, lanes A', B' and C'). In order to better characterize the Sgk1 dependent regulation of B-Raf further experiments were carried out in HEK293T cells over-expressing either wild type or mutant B-Raf [21]. Sgk1co-transfection significantly decreased B-Raf dependent ERK1/2 phosphorylation, in presence of wild type B-Raf. (Fig. 4 D, lane A') . Interestingly when HEK293T cells were transfected with a Ser364Ala B-Raf mutant lacking Ser364, that is the target of Sgk1 dependent inhibitory phosphorylation of B-Raf [21], a significant increase in ERK1/2 phosphorylation was

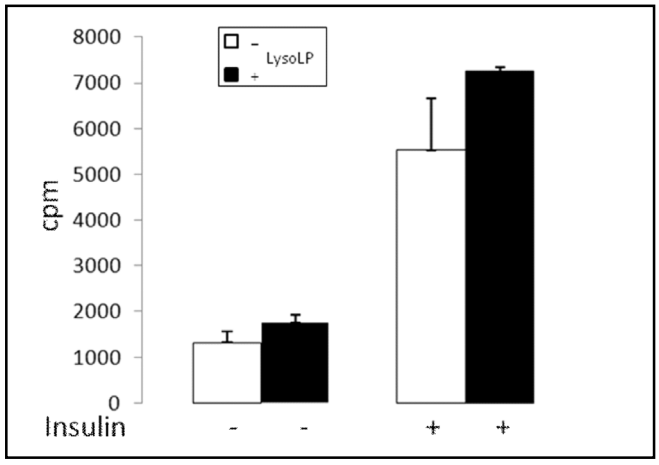

Fig 5. Sgk1 kinase activity, effects of insulin and LysoLP. HEK 293T cells transfected, with either pCMV-HA empty vector or vector coding for HA-LysoLP, were stimulated for 30 min with insulin (1 µmol). Endogenous Sgk1 was immunoprecipitated by Sgk1-specific immunoglobulins. The kinase activity associated with Sgk1 immunopreciptates was expressed as radioactivity (cpm) incorporated in an artificial substrate peptide as indicated in the materials and methods section.

detected (Fig. 4 D, lane K''), although this phosphorylation was not inhibited by Sgk1 co-transfection, thus demonstrating that Ser364 on B-Raf is indeed necessary for Sgk1 dependent B-Raf inhibition (Fig. 4 D, lane A'').

Sgk1 kinase activity

The heterologous expression of LysoLP was unable to affect the kinase activity. HEK293T cells, transfected with or without a vector coding for LysoLP, were treated with insulin, a well known activator of Sgk1 [4]. Endogenous Sgk1 was immunoprecipitated and used in an *in vitro* kinase assay with an artificial substrate and in the presence of [γ^{32}P]-ATP. Insulin consistently enhanced Sgk1 kinase activity whereas the overexpression of the LysoLP had no effect on either the basal or insulin-stimulated Sgk1-dependent kinase activity (Fig. 5).

The effect of LysoLP on ENaC activity

The effect of LysoLP on the regulation of the amiloride-sensitive epithelial sodium channel ENaC was studied in isolated *Xenopus* oocytes expressing ENaC in the absence or presence of LysoLP and Sgk1. (Fig. 6 A and B). ENaC-dependent current was increased by the expression of Sgk1, an effect totally reversed by coexpression of LysoLP (Fig. 6 A).

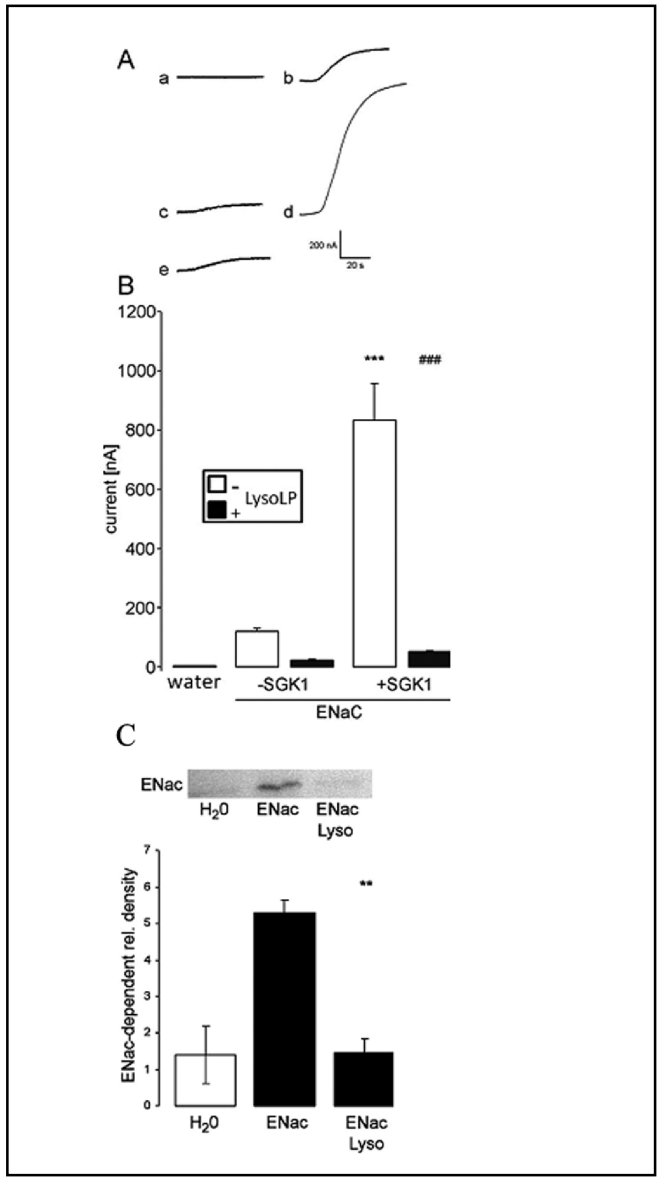

Fig 6. Experiments in Xenopus oocytes. A. Original tracings of the amiloride-sensitive ENaC-dependent current in *Xenopus* oocytes injected with water (a), expressing ENaC without (b, c) or with additional co-expression of Sgk1 (d, e), in absence (b, d) or presence of LysoLP (c, e). B. Arithmetic means ± SEM (n=10-17) of the amiloride-sensitive ENaC-dependent current in *Xenopus* oocytes injected with water, expressing ENaC without or with additional coexpression of Sgk1 in the absence (open bars) or presence (closed bars) of LysoLP. *** ($p<0.001$) indicates statistically significant difference from the absence of Sgk1. ### ($p<0.001$) indicates statistically significant difference from the absence of LysoLP. C. Membrane abundance of Flag-βENaC as detected by surface biotinylation (top). Bands were quantifed with Quantity One Software (bottom).

The LysoLP dependent inhibition of ENaC-dependent current was associated with a clear cut decrease in the membrane expression of ENaC, as

detected by surface biotinylation, thus demonstrating that the expression of LysoLP interferes with the membrane expression of ENaC (Fig. 6 C)

Discussion

Previous data obtained from A6 cells, a well-characterized model of the principal cells of the distal nephron, suggested that phosphatidylinositide-3-kinase (PI3K)-dependent phosphorylation of Sgk1 is essential for the activation of the enzyme and that it is indeed the active enzyme, which contributes to or even accounts for insulin-, vasopressin- and aldosterone-dependent stimulation of sodium transport through the amiloride-sensitive ENaC [23]. We performed yeast two-hybrid screening to identify molecular partners involved in the transduction of signals through Sgk1. We used a kidney library as a source of prey cDNAs because the hormonal regulation of sodium transport takes place in the principal cells of the distal nephron.

Using two-hybrid screening, we identified several independent clones coding for putative Sgk1-interacting proteins [12]. One of them, transportin (related to importin) has previously been shown by others to interact with the nuclear localization sequence of Sgk1 and to cause nuclear import of Sgk1 [24]. Another clone coding for PMM2, a key enzyme in protein glycosylation, has previously been characterized [12].

The 60 kDa Lysophospholipase (LysoLP) is a human clone similar to the rat liver lysophospholipase [15, 25]. Based on sequence analysis the enzyme has been proposed to be endowed with asparaginase activity, lysophospholipase activity and hydrolase activity, although the precise function is so far unknown. Recently, the official symbol ASPG has been attributed to the protein, standing for asparaginase homolog (*S. cerevisiae*) [Homo sapiens] GeneID: 374569.

Based on the yeast two-hybrid screening, co-immunoprecipitation and co-localization experiments, we present evidence for a physical interaction between Sgk1 and LysoLP. The use of an amino terminal mutant of LysoLP suggested that the N terminal portion of the molecule is critical for the interaction.

In order to elucidate the function of the new Sgk1-interacting protein, we started setting up asparaginase and lysophospholipase assays. We were unable to associate a consistent asparaginase activity with LysoLP (data not shown), whereas a mild, but significant, lysophospholipase activity was detected when lysophosphatidylinositol was used as substrate. This activity was not affected by Sgk1, indicating that although LysoLP and Sgk1 are physically associated, their interaction does not reciprocally affect their enzymatic activities.

It is worth remembering that Sgk1 has been demonstrated to negatively regulate B-Raf by inhibitory phosphorylation of Serine 364 [19] and is then expected to inhibit ERK phosphorylation as previously shown in a kidney cancer cell model [9]. This effect of Sgk1 is synergic with the effect of GILZ that enhances the activity of ENaC downregulating the tonic inhibition exerted by ERK phosphorylation [26], although the possible cross talk between Sgk1 and GILZ needs to be better defined. Indeed, a Sgk1-dependent inhibition of ERK1/2 phosphorylation was confirmed in our system. The use of the Ser364Ala B-Raf mutant demonstrates that Sgk1 dependent phosphorylation of Ser364 in B-Raf is required for the inhibition of Erk1/2 phosphorylation . Both the inhibition of ERK1/2 phosphorylation and the Sgk1-dependent stimulation of ENaC activity in *Xenopus* oocytes were reverted by co-transfection of LysoLP. Since we were unable to demonstrate an inhibitory effect of LysoLP on the kinase activity associated with Sgk1 we consider the possibility of a trapping interaction between LysoLP and Sgk1 that may result in sequestering Sgk1 away from its protein substrates. In fact the expression of LysoLP in frog oocytes appears to decrease significantly the membrane expression of ENaC, a feature that is tightly regulated by Sgk1 through phosphorylation of Nedd4-2 [27].

Taken together, these results are entirely consistent with previous studies showing that ENaC activity is decreased in response to increases in ERK activity [26, 28] and suggest a role for ERKs in the observed effects.

The lysophospholipase activity associated with LysoLP is indeed expected to have consequences on the lipid composition of cell membranes. The expression of LysoLP in HEK293T cells is expected to accelerate the conversion of lysophosphatidylinositol into glycerophosphoinositol, which is an intracellular messenger, derived from the Ras pathway and involved in the control of cell proliferation [29-31].

Modulation of the lysolipid content may have structural consequences at the level of the plasma membrane (decrease in membrane curvature), or, in the case of LysoPtdIns, can be part of the PI catabolism. On the other hand GroPIns is a molecule that is involved in several signal transduction pathways and has been considered an intracellular messenger in thyroid cells [32],

thus its Lyso-dependent formation may be part of a regulatory cascade yet to be uncovered.

The phosphoinositide metabolism plays a crucial role in transport [33] and has recently been demonstrated to participate in Sgk1 sensitive transport regulation [34]; both chelation of endogenous PtdIns45P_2 with anti-PtdIns45P_2 antibody and hydrolysis of endogenous via exogenous phospholipase C reduce ENaC activity [35]. In addition, PI3-kinase and the formation of PtdIns345P3 are required for basal, insulin- and aldosterone-stimulated sodium transport mediated via ENaC [36, 37].

More recently, the lipid composition of cell membranes has been demonstrated to be critically important for the Sgk1-dependent regulation of sodium transport through ENaC [36, 38]. Thus, changes in membrane lipid composition mediated by the LysoLP may also alter channel and transporter activities and may be responsible for the LysoLP-dependent inhibition of basal and Sgk1-stimulated sodium transport through ENaC.

In conclusion, we describe a novel molecular partner of Sgk1 that is similar to a previously identified rat liver lysophospholipase. Although the functional consequences of the interaction between Sgk1 and LysoLP remain unknown, these enzymes have independent and opposing effects on intracellular processes such as ERK1/2 phosphorylation and ENaC activity. Furthermore, these studies provide novel information on the function of a poorly characterized enzyme that is likely to be involved in the regulation of several Sgk1-dependent functions, related to cell proliferation and to the regulation of cell membrane channels.

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